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Translation of Human Hepatitis C Virus RNA in Cultured Cells Is Mediated by an Internal Ribosome-Binding Mechanism

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The human hepatitis C virus (HCV) contains a long 5′ noncoding region (5′ NCR). Computer-assisted and biochemical analyses suggest that there is a complex secondary structure in this region that is comparable to the secondary structures that are found in picornaviruses (E. A. Brown, H. Zhang, L.-H. Ping, and S. M. Lemon, Nucleic Acids Res. 20:5041–5045, 1992). Previous in vitro studies suggest that the HCV 5′ NCR plays an important role during translation (K. Tsukiyama-Kohara, N. Iizuka, M. Kohara, and A. Nomoto, J. Virol. 66:1476–1483, 1992). Dicistronic and monocistronic expression vectors, in vitro translation, RNA transfections, and deletion mutagenesis studies were utilized to demonstrate unambiguously that the HCV 5′ NCR is involved in translational control. Our data strongly support the conclusion that an internal ribosome entry site exists within the 5′ noncoding sequences proximal to the initiator AUG. Furthermore, our results suggest that the HCV genome is translated in a cap-independent manner and that the sequences immediately upstream of the initiator AUG are essential for internal ribosome entry site function during translation.

Hepatitis C virus (HCV) is the documented etiologic agent of blood-borne non-A, non-B hepatitis and represents the principal infectious agent associated with posttransfusion hepatitis (4). Recent clinical studies have demonstrated a strong correlation between HCV infection and hepatocellular carcinoma (28). The HCV genome is a single-stranded RNA molecule with plus-strand polarity and is approximately 9,500 nucleotides (nt) long (5, 13, 18, 24, 32). On the basis of sequence comparisons and in vitro translation studies, the HCV genome appears to encode several structural and nonstructural proteins (Fig. 1) (4, 12, 23). This information, in conjunction with additional characteristics, has led to the tentative classification of HCV in the family *Flaviviridae* (4, 27).

Eucaryotic mRNAs are translated by a mechanism known as ribosome scanning (19, 20). This mechanism involves binding of the 40S ribosomal subunit adjacent to the 5′ end of the mRNA, which is mediated by an interaction between the 5′ methylated cap structure and the cellular initiation factor complex eIF-4F. mRNA leader sequences, or the 5′ noncoding region (5′ NCR), play an important role during the translational regulation of viral and cellular RNAs. While these sequences are typically 50 to 100 nt long, longer 5′ leader sequences have been described. Members of the family *Picornaviridae*, such as poliovirus, encephalomyocarditis virus, and Theiler's murine encephalomyelitis virus (TMEV), contain noncoding sequences at the 5′ end of the genome that are 700 to 1,000 nt long. These sequences form multiple stem-loop structures, which contribute to a complex secondary structure that is capable of specifically binding viral and cellular proteins. For each of these picornaviruses, translation is initiated from an AUG codon that is located at an internal site several hundred nucleotides from the 5′ end of the genome (1, 15, 25). This mechanism involves binding of the ribosome to an RNA sequence that has been termed the internal ribosome entry site (IRES) (15), which is localized within the 5′ NCR of these viral RNAs. The IRES mechanism is distinct from the ribosome-scanning model, which predicts that translation is initiated from the AUG codon that is proximal to the 5′ end. In poliovirus, the IRES-initiated translation would give the virus a major selective advantage, since viral infection effectively inactivates the cellular cap-binding protein and leads to arrest of host cell protein synthesis (21).

HCV appears to contain a long, variable 5′ NCR which ranges from 332 to 341 nt, depending upon the HCV subtype. These sequences precede the AUG that is used for translational initiation of the HCV polyprotein (11, 13, 24, 32, 33). While several AUGs are located upstream from the translational initiation site, these AUGs do not appear to function as alternative start sites. Computer analyses of the HCV 5′ NCR, which is approximately half the length of the positive-strand RNA viruses in the family *Picornaviridae*, indicate the presence of a complex secondary structure (2). Recent in vitro studies have implicated the HCV 5′ NCR in translational control (34). The evidence presented in this report, based on the studies of transfection of cultured human cells and other relevant approaches, further supports the conclusion that the HCV 5′ NCR contains a functional IRES.

MATERIALS AND METHODS

Plasmid constructions. The 5′ noncoding sequences of the HCV genome, corresponding to nt 29 to 332, were amplified by the polymerase chain reaction (PCR) with plasmid BK146 (a generous gift of Hiroto Okayama, Osaka University, Osaka, Japan), which represents an HCV cDNA clone from a Japanese isolate (HCV-BK) (32). The sense primer 5′-CACTCCCCTGTGAGGAACTACTGT-3′ and the antisense primer 5′-CGAGCTCATGATGCACGGTC-3′ were used for PCR amplification. The PCR product representing the 5′

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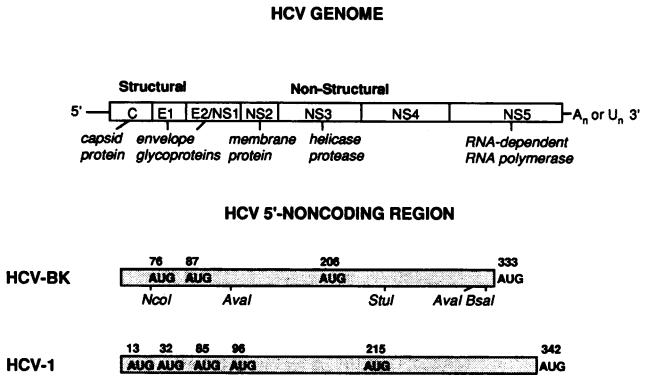

FIG. 1. Structure and organization of the HCV genome and the 5′ NCR of HCV-BK and HCV-1 strains. The locations of various AUG codons in the NCRs of the two HCV strains are indicated.

NCR of HCV was initially cloned into pGEM-3 to produce plasmid GHCV5′. The nucleotide sequences of GHCV5′ were determined by the dideoxynucleotide method. A full-length HCV 5′ NCR fragment was synthesized directly from RNA that was extracted from an HCV-positive serum sample obtained from the Denver, Colo., area. The viral RNA was reverse transcribed and PCR amplified with the sense primer 5′-GCCAGCCCCCTGATGGGGCGACACTCCAC CAT-3′, in which the first nucleotide represents the 5′-distal end of HCV-1 (11), and the antisense primer described above. Because the nucleotide sequences of this isolate at the 5′ NCR are homologous (about 99%) to the HCV-1 subtype, this isolate will be considered as equivalent to HCV-1.

A two-gene expression vector, T7-CAT/ICS/LUC, which contains the chloramphenicol acetyltransferase (CAT)- and luciferase (LUC)-encoding genes, was used to clone the 5′ noncoding sequences of HCV. Construction of T7-CAT/ICS/LUC has been previously described (22). The CAT-encoding gene was under control of the bacteriophage T7 promoter and was followed by an intercistronic spacer upstream of the LUC-encoding gene (Fig. 2A). The intercistronic spacer contains multiple cloning sites. The HCV 5′ noncoding sequences were inserted into the *SalI-NcoI* cloning sites to direct LUC expression from the dicistronic transcripts. Three recombinant plasmids containing the 5′ noncoding sequences derived from the HCV-BK strain, T7DC323-29, T7DC29-323, and T7DC29-332, were generated (Fig. 2B). Dicistronic expression plasmid T7DC1-341 contained the full-length 5′ NCR derived from HCV-1. PCR-amplified synthesis of the 5′ NCR from this HCV isolate is described above. The numbers in these and all other plasmids refer to the exact nucleotide sequences of the 5′ NCR. In plasmid T7DC29-323, nt 29 to 323 (sense orientation) were cloned into T7CAT/ICS/LUC by blunt-end ligation. Plasmid T7DC323-29 contains the same sequences in the antisense orientation. In plasmid T7DC29-332, a nearly full-length 5′ NCR (nt 29 to 332) was inserted into T7CAT/ICS/LUC in the sense orientation at the *SalI-NcoI* sites.

A series of plasmid vectors were generated for synthesis of monocistronic RNAs containing various lengths of the HCV 5′ NCR in front of the LUC-encoding gene. Plasmid T7-LUC (22), which contained the *SalI-NcoI* cloning sites between the T7 promoter and the LUC-encoding gene, was used for insertion of HCV 5′ noncoding sequences (see Fig. 4A). For the structures of these plasmids, see Fig. 4B.

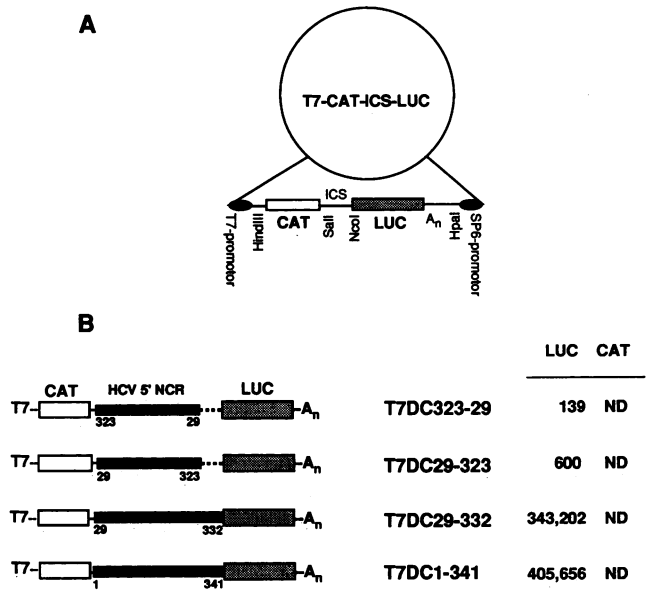

FIG. 2. Structures of dicistronic expression vectors. (A) Structure of the parent plasmid, T7-CAT/ICS/LUC (22), used for construction of the plasmids described in panel B. The locations of the promoters and the relevant restriction endonuclease cleavage sites are shown. ICS, intercistronic spacer. (B) Structures of the HCV 5′ NCR-containing constructs. 5′ noncoding sequences of HCV (solid bar) were cloned into the intercistronic spacer site in the sense and antisense orientations as indicated. The relative LUC activity (light units per 5×10^5 cells) of each construct (10 μg) in transfected HepG2 cells is shown. ND, not detectable.

Fragments representing various HCV 5′ noncoding sequences were cloned into T7-LUC with blunted or cohesive termini.

In vitro transcription and translation. Plasmid DNAs were linearized with *HpaI* and then transcribed in vitro with T7 RNA polymerase (New England BioLabs) in accordance with the manufacturer's instructions. To generate methylated cap RNAs, ^{7}mGpppG was included in the reaction. In vitro translation was done with rabbit reticulocyte lysates (Promega). Proteins were labeled with Tran^{35}S-label (ICN Biomedicals) and subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein gels were dried and exposed to X-ray film.

Cell culture and viral infection. HepG2 cells, a human hepatoblastoma cell line, were used in this study for transfections and poliovirus infections. HepG2 cells were infected with poliovirus (type I, Mahoney) at a multiplicity of infection of 10 as described previously (10).

RNA transfection. In vitro-synthesized RNAs were introduced into cultured cells with Lipofectin (GIBCO/BRL). Nearly confluent cells in 60-mm-diameter petri plates were transfected with 30 μg of Lipofectin mixed with 5 to 10 μg of RNA as described previously (1). At 5 h posttransfection, cell lysates were prepared and assayed for either CAT (30) or LUC expression (7). Poliovirus-infected cells were transfected with RNA at 3.5 h postinfection and harvested 2 h later.

RNA stability. To determine the stability of transfected RNAs, an RNase protection assay was employed (29). At 5 h posttransfection, cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (3). A ^{32}P-labeled RNA probe was prepared from a T7-LUC plas-

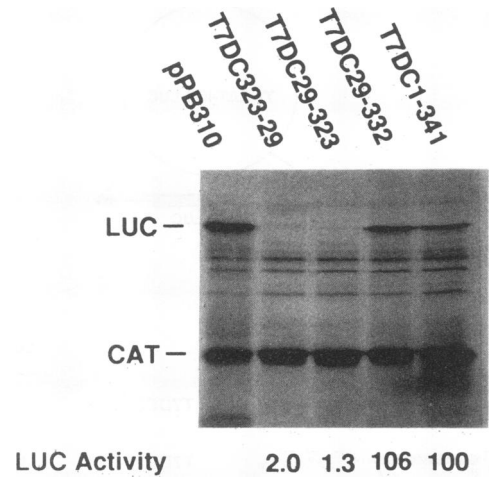

FIG. 3. In vitro translation of dicistronic RNAs in rabbit reticulocyte lysates. The positions of LUC and CAT proteins are marked. The numbers at the bottom show percentages of LUC activity normalized against the value obtained with T7DC1-341, which was arbitrarily set at 100. Plasmid pPB310 contains the entire 5′ NCR of TMEV (1) and was used as a positive control.

mid with a truncated LUC-encoding gene. The probe consisted of 420 nt derived from the modified plasmid, which was synthesized in vitro with SP6 RNA polymerase (Promega) in the presence of [α-^{32}P]CTP (Amersham). The probe contained 360 nt that were complementary to the monocistronic LUC RNAs used in the transfection studies.

RESULTS

The 5′ NCR of the HCV genome mediates translation of the second cistron in dicistronic RNA both in vitro and in vivo. To study the role of the HCV 5′ NCR during translation, we utilized a two-gene expression strategy (22, 25). For this approach, the 5′ NCR was inserted between two reporter genes, those for CAT and LUC (Fig. 2A). Since the T7 promoter precedes the CAT gene in these constructs, T7 RNA polymerase was used to synthesize the dicistronic transcripts. These transcripts have the following general structure: 5′-CAT-5′ NCR-LUC-An. The expression of the LUC gene from these RNAs is dictated by the HCV 5′ NCR. The full-length 5′ NCR (T7DC1-341) (HCV-1) and several truncated versions of the sequence from the HCV-BK subtype were analyzed (Fig. 2B).

In vitro-synthesized, uncapped dicistronic RNAs were translated in rabbit reticulocyte lysates. Figure 3 demonstrates that the CAT translation product was expressed at approximately equivalent levels for all of the dicistronic constructs. However, expression of the second cistron (LUC) varied dramatically. Dicistronic constructs T7DC1-341 and T7DC29-332 were extremely efficient in supporting LUC protein synthesis. LUC synthesis was inefficient in the presence of the antisense 5′ NCR (T7DC323-29). An additional truncation of nine nucleotides at the 3′ end of the sense 5′ NCR (T7DC29-323) dramatically affected translation of the LUC gene (Fig. 3).

Dicistronic RNAs were subsequently used to transfect cultured cells. HepG2, a hepatoma cell line, was used for RNA transfection studies by using the Lipofectin method. Maximal LUC activity was observed at 4 to 6 h posttransfection. Transfected cells were harvested at 5 h posttransfection and assayed for CAT and LUC activities. Since the in vitro-synthesized RNAs were uncapped, the first cistron encoding the CAT protein was poorly translated in transfected cells. CAT activity was not detectable in the cell lysates (Fig. 2B). However, expression of the second cistron (LUC) occurred only when the HCV 5′ NCR was present upstream of the cistron. As indicated in Fig. 2B, both the full-length (T7DC1-341) and the nearly full-length (T7DC29-332) 5′ NCRs were the constructs efficient in programming expression of the LUC cistron. Since this expression occurred independently of CAT translation, it is unlikely that LUC expression resulted from ribosome reinitiation. Similar results were obtained with transfected HeLa cells (data not shown). Therefore, there do not appear to be liver cell-specific preferences in translational efficiency. These data, which are derived from in vitro translation and RNA transfection studies, suggest that the 5′ noncoding sequences of HCV between nt 29 and 332 are capable of directing translation of the second cistron in a dicistronic RNA and that an IRES element is likely to be present within this region.

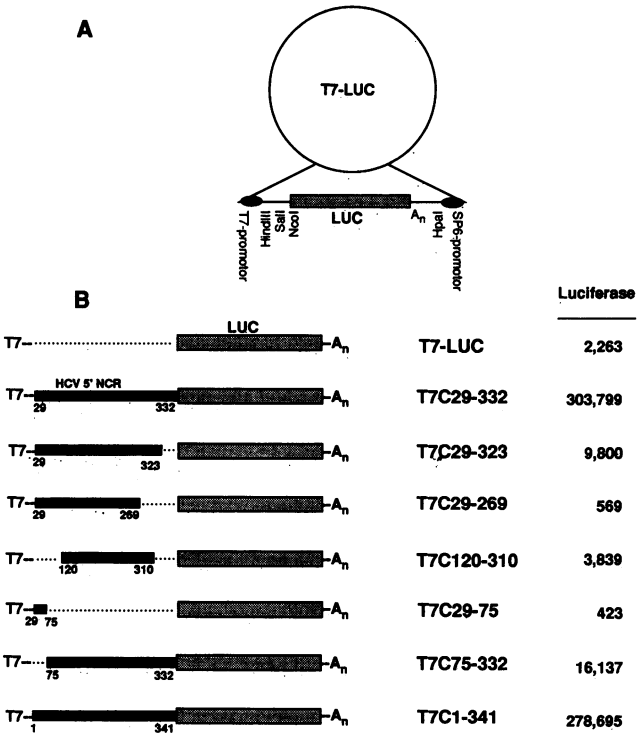

FIG. 4. Structures of DNA constructs used for synthesis of monocistronic RNAs. (A) Structure of T7-LUC (22). (B) Structures of HCV 5′ NCR-containing constructs. Various lengths of the HCV 5′ NCR (solid bars) were inserted between the T7 promoter and the coding sequences of the LUC gene in T7-LUC. LUC activity is expressed as light units per 5 × 10^5 cells transfected with 10 μg of each RNA.

Identification of IRES. The studies described above have established the functional role of the HCV 5′ NCR in internal initiation of translation in a dicistronic expression system. To define further the minimal sequences within the 5′ NCR of HCV that serve as an IRES, we generated a series of deletions in the 5′ NCR of the HCV-BK subtype. The constructs included both 5′ and 3′ end deletions of the 5′ NCR, which were then cloned in front of the LUC gene (Fig. 4B).

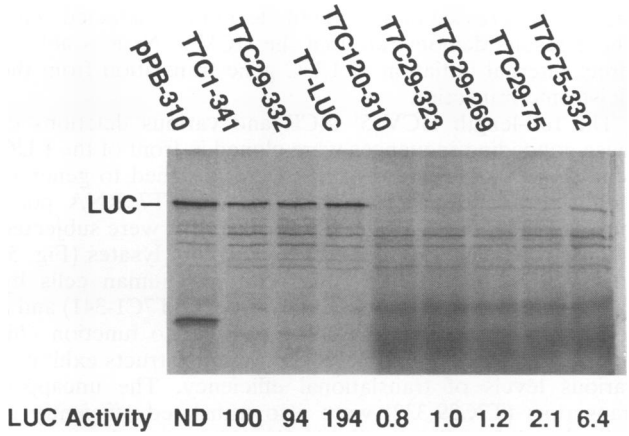

FIG. 5. In vitro translation of monocistronic RNAs in rabbit reticulocyte lysates. SDS-gel pattern of [^{35}S]methionine-cysteine-labeled translation products with various T7-derived transcripts (Fig. 4B). The numbers at the bottom show the percentages of LUC activity from individual lysates normalized against T7C1-341, which was arbitrarily set at 100.

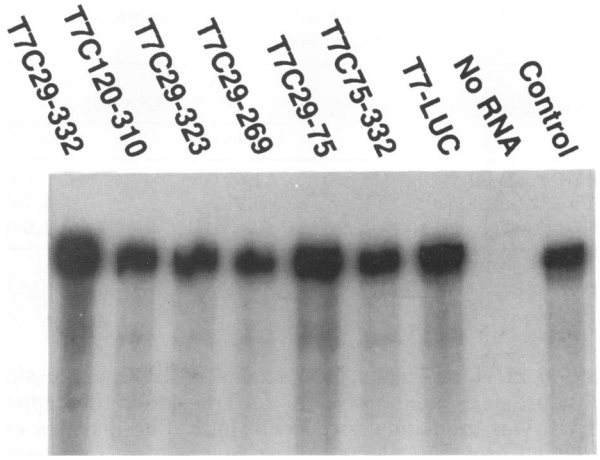

FIG. 6. Quantitation of the LUC reporter RNA in transfected HepG2 cells by RNase protection assay (see Materials and Methods). Protected RNAs were analyzed by urea-PAGE. Mock-transfected cells were used as the negative control (no-RNA lane), and in vitro-synthesized T7-LUC RNA was used directly in the assay as the positive control (control lane).

In vitro-synthesized monocistronic RNAs were translated in vitro in a rabbit reticulocyte lysate system (Fig. 5). [^{35}S]methionine-cysteine-labeled LUC expression was substantially pronounced when T7C1-341- and T7C29-332-derived transcripts were used. The translational efficiency of the HCV 5′ NCR, as represented by constructs T7C1-341 and T7C29-332, was comparable to that of a control construct containing a TMEV 5′ NCR (pPB310) (1). TMEV, a member of the family *Picornaviridae*, putatively contains an IRES within its 5′ NCR (1). Various HCV 5′ NCR deletion mutants inefficiently translated the LUC gene. Interestingly, T7C75-332-derived RNA displayed less stimulation of LUC expression. The LUC activity present in these lysates, which is indicated at the bottom of Fig. 5, correlates with the SDS-polyacrylamide gel pattern of the LUC protein.

RNA transfections were done with HepG2 cells with the monocistronic RNAs (Fig. 4B). The LUC gene was most efficiently expressed in the presence of both the full-length (T7C1-341) and the nearly full-length (T7C29-332) RNA constructs. In this case, translation was stimulated 100-fold compared with the value obtained with T7-LUC RNA. This level of activity was markedly reduced in the presence of various deletions of the 5′ noncoding sequences. For example, T7C29-75 and T7C29-269 mediated background levels of LUC gene expression. Similar results were obtained with transfected HeLa cells (data not shown). It is important to indicate that all of these RNAs were uncapped. Therefore, our findings, which have demonstrated that the HCV 5′ NCR mediates translation of the LUC gene, strongly suggest that an IRES exists within the 5′ NCR. This implies that HCV translational regulation functions in a cap-independent manner. It may be concluded from these results that sequences between nt 29 and 332 are essential for efficient IRES function, perhaps by generating a complex secondary structure which facilitates the processes of internal ribosome binding.

Stability of RNAs in transfected HepG2 cells. To rule out the possibility that RNA instability accounts for the differences in expression of the LUC cistron in transfected cells (Fig. 4B), RNase protection assays were performed. The results of such experiments are shown in Fig. 6. This analysis demonstrates that all of the monocistronic RNAs exhibited approximately equal levels of stability, whereas their translation was variable, as described above (Fig. 4B).

IRES of the HCV 5′ NCR is active in poliovirus-infected HepG2 cells. Poliovirus translation occurs in a cap-independent manner. The viral infection also results in shutoff of host protein synthesis. Cleavage of p220, a component of eIF-4F, has been suggested as a possible mechanism which initiates these events (21). To determine whether the HCV 5′ NCR could function in a similar manner, we infected HepG2 cells with poliovirus. The effect of poliovirus infection upon host cellular protein synthesis is presented in Fig. 7. At 3.5 h after poliovirus infection of HepG2 cells, most of the proteins expressed were virus encoded (Fig. 7, lanes 4 to 6). RNAs were introduced at 3.5 h subsequent to poliovirus

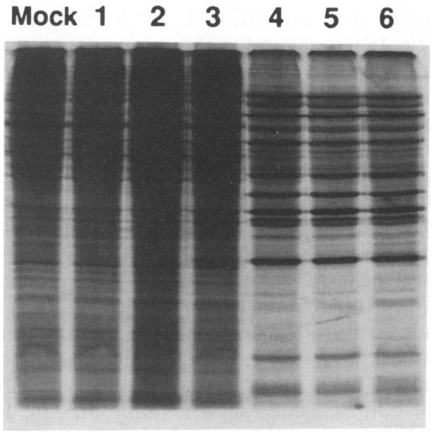

FIG. 7. Time course of shutoff of host cell translation after poliovirus infection. Cultured HepG2 cells were mock infected or infected with poliovirus at a multiplicity of infection of 10. At 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 h postinfection, the cells were pulse-labeled with [^{35}S]methionine-cysteine for 30 min. At 1, 2, 3, 4, 5, and 6 h postinfection, the cells were lysed and fractionated by SDS-PAGE (10) (lanes 1 to 6, respectively).

TABLE 1. Translation of RNAs in poliovirus-infected and uninfected HepG2 cells

RNA[a]	LUC activity (light units/ 5×10^5 cells)[b]	
	Uninfected	Infected
T7-LUC (capped)	1,672	190
T7C29-332 (uncapped)	3,973	1,702
pPB310 (uncapped)	2,626	1,081

[a] A 1-μg sample of each was used.
[b] These data represent the results of several experiments.

infection of HepG2 cells. The results of LUC expression assays are presented in Table 1. Translation of capped T7-LUC was dramatically reduced, indicating that this experimental protocol selects for cap-independent translation of mRNAs. Translation of uncapped T7C29-332 transcripts occurred efficiently in infected cells, strongly supporting the conclusion that cap-independent translation is associated with the 5′ NCR of HCV. A slight decrease in LUC expression of T7C29-332 RNA in poliovirus-infected cells was most likely due to competitions with polioviral IRES-containing RNAs for the translational apparatus. Similar decreases were observed with transcripts of pPB310, which contains the 5′ NCR of the picornavirus TMEV. Taken together, these results suggest that HCV utilizes a translational regulatory mechanism that is similar to that described for picornaviruses.

DISCUSSION

HCV represents the major viral agent of blood-borne non-A, non-B hepatitis. The RNA genome of this virus contains a long stretch of 5′ noncoding sequences. There are several AUG codons within this stretch of over 300 nt. Some of these AUG codons appear to be present within the sequence context that is favorable for translation. In the HCV-BK strain that was used in this study, translation is initiated from an AUG codon that is located at nt 333 (nt 342 for HCV-1). The sequences downstream of this AUG encode the large viral polyprotein. The studies presented in this report indicate that the HCV 5′ NCR contains an IRES element. The IRES regulates initiation of translation from the authentic AUG by an internal ribosome-binding mechanism, which is distinct from the linear scanning model. These conclusions are supported by data derived from several experimental approaches.

We utilized a synthetic dicistronic RNA containing the HCV 5′ NCR, which was inserted between the upstream CAT and downstream LUC genes (Fig. 2). Expression of the LUC cistron was analyzed by both in vitro translation (Fig. 3) and RNA transfections of cultured human hepatoma cells (Fig. 2). The RNA transfection technique is a powerful approach for analysis of translational regulation. For the RNA transfection studies, synthetic RNA molecules that were generated by T7 RNA polymerase were introduced directly into cultured cells with Lipofectin. The Lipofectin method is known to minimize RNA degradation. RNA molecules that reach the cytoplasm are translated in a cap-independent manner. Expression of the LUC gene from the dicistronic construct is dictated by the HCV 5′ NCR sequences. Cells that were transfected with these RNAs expressed the LUC gene independently of the CAT gene. The CAT gene, whose mRNA was devoid of a 5′ methylated cap, was expressed at a negligible level in transfected cells. These results demonstrate that the HCV 5′ NCR is able to direct internal initiation of LUC gene translation from the dicistronic transcript.

The full-length HCV 5′ NCR and various deletions of these noncoding sequences were cloned in front of the LUC gene (Fig. 4). These constructs were designed to generate monocistronic transcripts, in vitro, with T7 RNA polymerase. The monocistronic LUC transcripts were subjected to in vitro translation in rabbit reticulocyte lysates (Fig. 5) and were also introduced into cultured human cells by Lipofectin (Fig. 4). The full-length 5′ NCR (T7C1-341) and a short truncation (T7C29-332) were found to function efficiently. However, the 5′ NCR deletion constructs exhibited various levels of translational efficiency. The uncapped transcripts (T7C29-332) were also translated efficiently in poliovirus-infected cells (Table 1), which strongly suggests that the HCV genome is translated in a cap-independent manner.

Deletion analysis of the 5′ noncoding sequences was done to delineate the minimal sequence requirement for internal initiation at the 5′ end of the 5′ NCR. This analysis indicated that deletion of up to 75 nt from the 5′ end dramatically reduced the efficiency of translational initiation. The 5′ end deletions beyond nt 75 were totally inefficient in initiating translation from an internal site. This result is in contrast to the work of Nomoto et al. (34), which indicated that deletion of 101 nt from the 5′ end did not adversely affect efficiency of translation. The reason for this descrepancy is unclear. Deletion of sequences from the 3′ end of the 5′ NCR also abrogated translational initiation from an internal site. These findings suggest that sequences immediately upstream of the initiator AUG codon play a key role in determining the translatability of the HCV genome. These sequences exhibit a secondary structure that is indicative of a stable stem-loop structure (2). Such a structure may serve as a ribosome landing pad that is essential for internal binding of ribosomes. Given this possibility and the occurrence of efficient and accurate HCV translation in rabbit reticulocytes lysates, HCV may share structural and functional similarities with the cardioviruses, such as encephalomyocarditis virus and TMEV. Poliovirus and rhinoviruses translate relatively inefficiently and inaccurately in rabbit reticulocyte lysates (8).

Yoo et al. reported that the full-length 5′ NCR derived from the HCV-1 isolate failed to direct translation, while sequential deletions in the 5′ NCR were found to mediate translation (35). They argued that a hairpin structure at the 5′ end of the 5′ NCR prevented translation. A deletion mutant lacking this structure, however, was found to be efficient in translation. To address this point, we synthesized a full-length 5′ NCR by PCR amplification. This full-length 5′ NCR (nt 1 to 341), in the contexts of both dicistronic and monocistronic expression vectors, mediated translation as efficiently as the 5′ NCR construct containing nt 29 to 332 of the HCV-BK strain (Fig. 2B, 3, 4B, and 5). We show here that constructs T7DC1-341 and T7C1-341, containing the full-length 5′ NCR (HCV-1), are fully functional for translational initiation. It is unlikely that the hairpin structure at the 5′ end of the HCV genome functions to inhibit translation. This structure is also present in polioviral RNA yet does not appear to affect translational efficiency adversely (31). Moreover, our analysis of the intact, full-length 5′ NCR (T7C1-341) allays that concern (Fig. 4B and 5).

The constructs utilized by Yoo et al. (35) contained approximately 30 additional nt between the initiator AUG and the extreme 3′ end of the HCV 5′ NCR. The additional

nucleotides, which were derived from the CAT gene, may have had an anomolous effect upon those studies. This is especially important since our results indicate that the sequences immediately upstream of the initiator AUG are critical for the integrity of 5′ NCR-mediated translational control. It has been suggested that in encephalomyocarditis virus, the ribosome binds directly or very closely to the initiator AUG (14, 16, 17), and the AUG appears to be involved with internal ribosome entry (26). The distance between the AUG and the upstream motif may be important for IRES efficiency. Interestingly, deletion or addition of nucleotides upstream of the AUG decreased or abolished encephalomyocarditis virus IRES function (6, 16). In our constructs and those of Nomoto et al. (34), the 3′ end of the HCV 5′ NCR was inserted immediately adjacent to the AUG of the reporter gene. Brown et al. (2) have predicted that a secondary structure in that region brings the initiator AUG to within close proximity of a characteristic pyrimidine-rich tract. If such structures play important roles in translational regulation by generating ribosome landing pads, or an IRES, the presence of extraneous nucleotide sequences in that region may dramatically alter the ability of these sequences to support internal initiation of translation. This model predicts that the additional nucleotide sequences will perturb stem-loop structure IV, located upstream of the initiator AUG (2). Therefore, the presence of additional sequences (30 nt) in the constructs used by Yoo et al. (35) probably destroyed stem-loop structure IV, thus adversely affecting the functional IRES. Obviously, this hypothesis can be tested experimentally.

We have further noted that the monocistronic transcripts used by Yoo et al. (35) included a 5′ cap structure. In our studies, while the cap structure at the 5′ terminus of the HCV 5′ NCR could support translational initiation from the internal site, uncapped transcripts were more efficient (data not shown). On the basis of this finding, in conjunction with our studies which have demonstrated that uncapped HCV monocistronic transcripts are translated efficiently in poliovirus-infected cells, we propose that HCV translation occurs in a cap-independent fashion. Similar conclusions were also reached by Nomoto and coworkers (34). It has been shown that the 5′ capped poliviral 5′ NCR does not initiate translation efficiently in transfected cells (9). In light of the concerns discussed above, we believe that our findings differ from those reported by Yoo et al. (35) because of the use of disparate constructs.

In summary, this report presents evidence from several experimental approaches which strongly argues for the presence of an IRES element in the HCV 5′ NCR. Furthermore, the IRES regulatory element appears to mediate translation of the HCV genome by a mechanism(s) that is similar to that utilized by the viruses of the *Picornaviridae* family.

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